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Fundamentals, achievements and challenges in the electrochemical sensing of pathogens.

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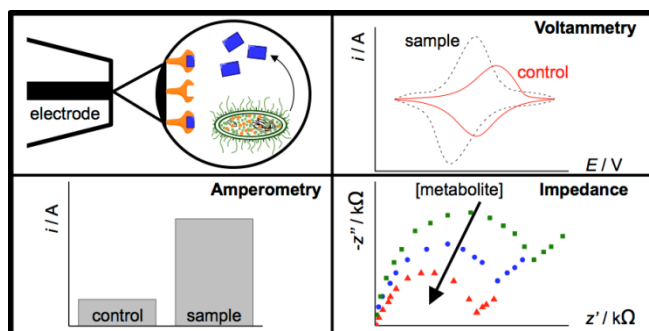
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Abstract

Electrochemical sensors are powerful tools widely used in industrial, environmental and medical applications. The versatility of electrochemical methods allows for the investigation of chemical composition in real time and *in situ*. Electrochemical detection of specific biological molecules is a powerful mean for detecting disease-related markers. In the last 10 years, highly-sensitive and specific methods have been developed to detect waterborne and foodborne pathogens. In this review, we classify the different electrochemical techniques used for the qualitative and quantitative detection of pathogens. The robustness of electrochemical methods allows for accurate detection even in heterogeneous and impure samples. We present a fundamental description of the three major electrochemical sensing methods used in the detection of pathogens and the advantages and disadvantages of each of these methods. In each section, we highlight recent breakthroughs, including the utilisation of microfluidics, immunomagnetic separation and multiplexing for the detection of multiple pathogens in a single device. We also include recent studies describing new strategies for the design of future immunosensing systems and protocols. The high sensitivity and selectivity, together with the portability and the cost-effectiveness of the

instrumentation, enhances the demand of further development in the electrochemical detection of microbes.

Visual Abstract



1. Introduction

Even though numerous analytical methods are used to identify and quantify pathogenic agents, these methods still depend on conventional culturing techniques and must be performed in a microbiology laboratory.¹ Therefore, these techniques lack versatility and portability and cannot be used for on-site monitoring.² In addition, conventional culturing techniques are time-consuming and require trained personnel, thus compromising a timely response to possible outbreaks.

A great research challenge in this field is therefore to develop rapid, reliable, specific, and sensitive methods to detect these pathogens at low cost.^{3,4} In a broad range of applications, electrochemical sensors have been extensively investigated and have proved to be inexpensive and sensitive methods for the detection of the analytes involved in healthcare, environmental monitoring, food packaging and many other applications.^{5,6} One of the best examples of electrochemical sensors is the low-cost and portable glucose blood sensor

strips. This technology, based on the amperometric sensing of glucose using screen-printed enzyme electrodes, has generated revenues of over \$5 billion/year.⁷

For those interested in the use and development of electrochemical tools for sensing bacteria, this review aims to provide a first point of contact, emphasising fundamental sensing principles and advances in materials, characteristics and performance. In the first section, we describe the principles behind electrochemical sensing, current electrochemical methods and their advantages and disadvantages. In the second section, we provide a selection of case studies of the application of these electrochemical methods for detecting pathogenic bacteria. Such case studies will simply illustrate the strategies implemented to improve sensitivity, selectivity and portability, rather than provide a comprehensive review of the current literature in the area. Interested readers are encouraged to further their interest with other reviews^{4-6,8-15} which extensively cover individual aspects of pathogen detection, in-depth descriptions of some of the techniques discussed below,^{8,9} the use of metal nanoparticles (NP),^{10,12,13} and the use of multiplexing¹¹ or screen-printed electrodes.^{6,14,15} In the final section, we provide a general view of the challenges faced when developing an electrochemical sensor that can deliver a fast, reliable and sensitive response to the presence of a bacterial pathogens. Solving these challenges will require the combined expertise of electrochemists, biomedical scientists, synthetic chemists, material scientists and electronic engineers.

1.1. The challenge of early detection of pathogenic bacteria

Bacterial infections remain a leading cause of morbidity and mortality worldwide.¹⁶ This is especially the case in developing nations where infectious diseases cause up to 40% of

deaths.¹⁷ Poor sanitation or lack of access to treatment are among the main causes for the high impact of bacterial infections in these countries. In recent times, new challenges are emerging that affect developed countries as well: there is an increase in infectious bacteria that are resistant to our current antibiotics and antimicrobials (Figure 1),^{18,19} while the pipeline of novel antibiotics that can target these resistant bacteria is currently very limited.²⁰ As such, the landscape for infectious diseases in developed countries is likely to change in the forthcoming years.

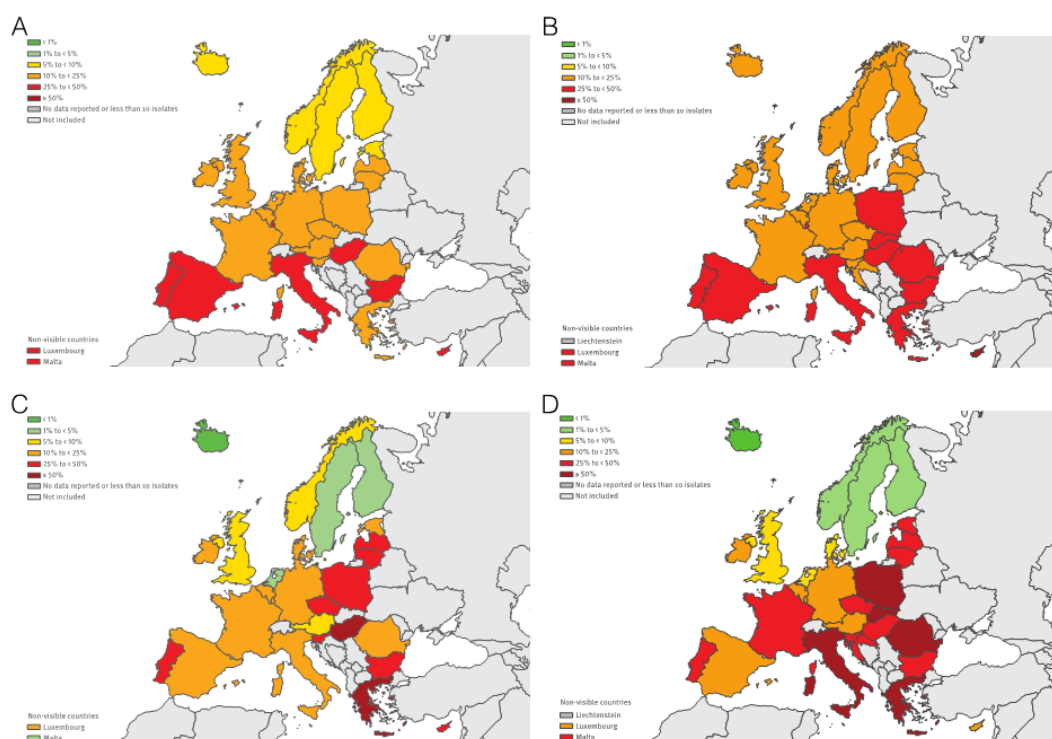


Figure 1: Proportion of invasive *Escherichia coli* isolates resistant to fluoroquinolones in 2009 (A) and 2013 (B). Data for *Klebsiella pneumoniae* in 2009 (A) and 2013 (B).^{18,19}

Of all the routes of infection, water and the food chain are still the fastest modes of transmission.²¹⁻²⁵ In addition, there is an increase in the number of healthcare-associated infections, especially of resistant strains of opportunistic microbes (e.g. *Pseudomonas*

aeruginosa, *Klebsiella*, *Acinetobacter*). These emerging pathogens pose new challenges, the least of which is the need to develop rapid and point-of-care diagnostics. We need to be able to quickly and reliably identify the causative agent of infection and its antimicrobial resistance so that tailored treatments can be implemented which selectively target the desired pathogen. Targeted treatment is essential to minimise the unwanted effects of broad-spectrum antimicrobials; in particular, the increase in antimicrobial resistance.

2. Electrochemical methods

This section describes the fundamentals of the three common electrochemical methods used for detecting pathogens: 2.1) voltammetry, 2.2) amperometry/potentiometry and 2.3) impedance spectroscopy. We include a general description of how different analytes can be sensed using these electrochemical methods. In addition, we highlight their fundamental advantages and limitations to help the reader select the best method for a specific analysis.

2.1. Voltammetric detection

Voltammetric methods are based on the change in the potential that is applied to the electrode-solution interface as a function of time, together with the measurement of the current.²⁶ Cyclic sweep voltammetry is often used as the preferred method for the acquisition of information such as oxidation/reduction potential, kinetics, and reaction mechanisms.^{26,27} As can be seen from Figure 2A, in a cyclic voltammetry, the voltage is scanned from a lower limit potential to an upper limit potential. The scan rate is defined by the time taken to sweep the whole potential range; the current response is plotted as a function of voltage rather than time. Two different sensing approaches can be followed when using cyclic voltammetry: 1) receptor mediated sensing (Figure 2B), and 2) direct

oxidation/reduction reaction of the metabolite on bare (i) or functionalised electrodes (ii)
(Figure 2C).

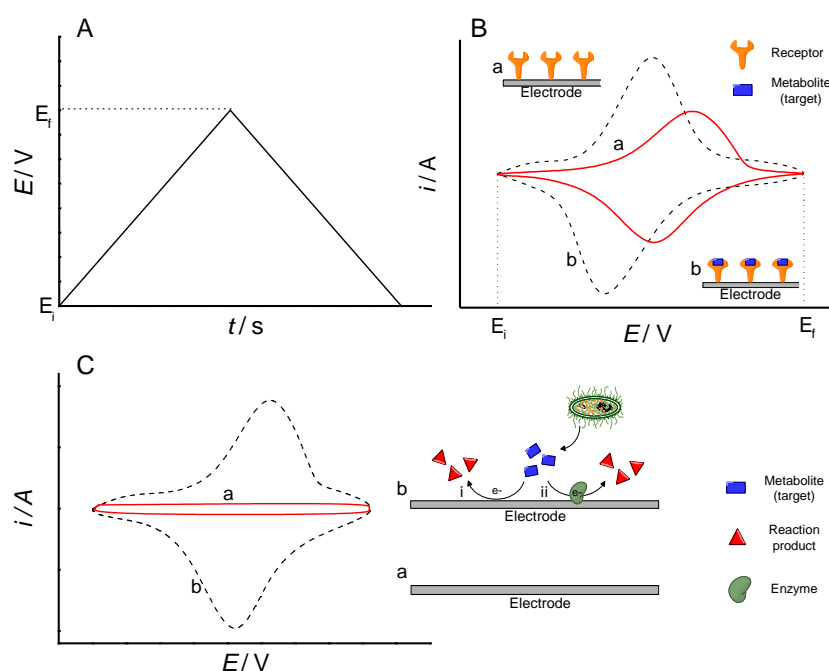


Figure 2: (A) Cyclic voltammetry waveform showing the initial potential (E_i) and the switching potential described by the maximum (E_f). (B) Cyclic voltammogram of a receptor-coated electrode (a) where its electrochemical response varies upon specific metabolite binding (b). (C) Voltammetric profile of a bare electrode (a) and the electrochemical detection of bacterial metabolites (b): based on their redox reaction on plain electrodes (i) or their enzyme-catalysed conversion on specifically functionalised electrodes (ii).

Amongst all the electrochemical characterisation methods, pulse strategies, such as Differential Pulse Voltammetry (DPV), Normal Pulse Voltammetry (NPV) and Square Wave Voltammetry (SWV), are perhaps the most sensitive and therefore most extensively used in

electro analysis. The main advantage of pulse techniques resides in the different decay rates of the charging and faradaic currents. Because the charging current decays much more rapidly than the faradaic current, during each pulse, the capacitive current is negligible compared to that of the faradaic current.²⁶ This increased ratio between the faradaic current with respect to the capacitive current allows for a lower detection limit, ideal for analytical purposes. DPV (Figure 3A) and SWV (Figure 3B) are often the first choice in electroanalysis due to their ability to be operated at high frequency, thus providing a unique time resolution component not present in the other pulse techniques.⁹ Another significant advantage of these methods is the possibility for canceling the oxygen reduction currents (or other residual currents) from the background.^{27,28} There are numerous studies related to electrochemical sensing of pathogens using SWV.^{9,29,30} The advantage of SWV over DPV is the quick response, even at high effective scan rates (1 V/s), which reduces the scan time of the analysis. In addition, SWV has a lower consumption of electroactive compounds in relation to DPV and has fewer complications related to the blocking of the electrode surface. Figure 3C shows the typical SWV response of an electrode, modified with a specific receptor, as a function of the concentration of a metabolite produced by a bacterial pathogen.

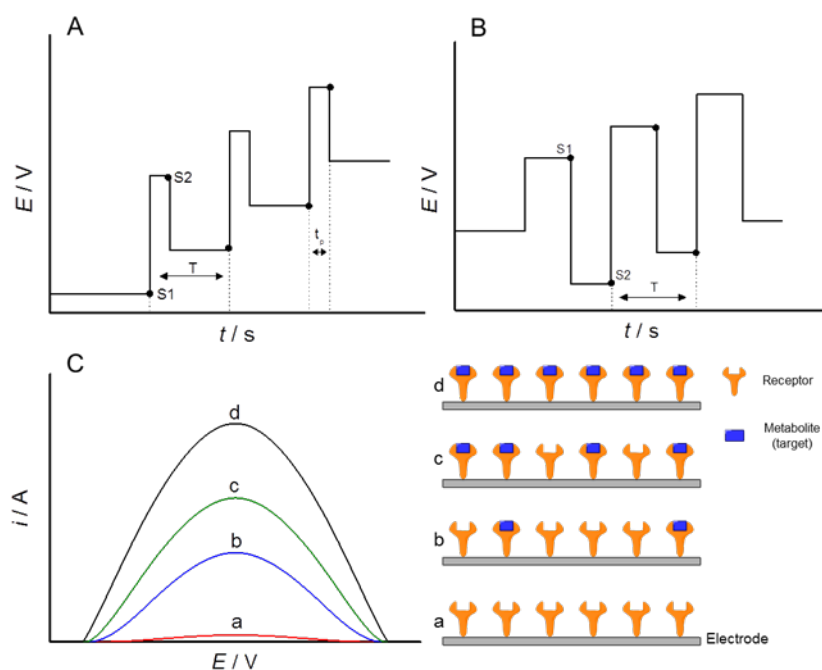


Figure 3: (A) Potential-time profile of DPV. (B) Potential-time profile of SWV. (C)

Representation of metabolite detection by means of receptor-coated electrodes: current variations are proportional to receptor occupancy. Initial potential ($S1$), potential after the pulse ($S2$), pulse frequency (T) and pulse duration (t_p).

2.2. Amperometric and potentiometric detection

Amperometric and potentiometric detection of pathogens are based on the changes in current (or potentials) while the electrode is maintained at a constant potential (or current) with respect to the reference electrode.²⁶ In both cases, the sensor consists of biological structures that can selectively adsorb microbes (*e.g.* glycans, antibodies or aptamers), coupled to an enzymatic transduction system. The enzyme-catalysed reaction is only triggered upon bacteria adsorption and generates or consumes a species, which is subsequently detected by an ion-selective electrode.²⁸

These methods provide a logarithmic concentration dependence, which implies high sensitivity. The recent implementation of field effect transistors for the detection of microbial contamination amplified the signal of the analytical device, providing improved sensitivity in a reduced circuit. A field effect transistor is a semiconductor device used to amplify and switch electronic signals.^{31,32} In addition to the higher sensitivity, these detection methods are also time-effective and economical.^{28,33} However, amperometric sensors suffer from poor selectivity. This is because all components in solution with a standard potential, E° , smaller than the operating potential, E , of the sensor will contribute to the faradaic current.

2.3. Impedimetric detection

This type of sensor detects impedance changes to small amplitude, sinusoidal voltage signal as a function of frequency, in the dielectric properties of an electrode upon analyte adsorption.^{26,28} Two strategies have been used for the detection of pathogens using impedimetric techniques. In one strategy, bare electrodes, or electrodes modified with bioreceptors, can be used to measure the impedance change caused by the unspecific, or specific binding of bacteria to the electrode, respectively (Figure 4).⁸ The second strategy directly detects metabolites produced by pathogens as a result of growth – a powerful tool for sensing bacterial toxins in contaminated samples (Figure 5).^{34,35}

Impedimetric methods can quantify the extent to which the bacterium or metabolite is bound to the electrode (functionalised or not). Such interaction is measured through either a change in the magnitude of the capacitance at the electrode interface, or a shift in impedance (Figure 4). The bacterial cell membrane consists of a phospholipid bilayer with

thickness between 5 and 10 nm. This membrane has a typical capacitance of $0.5\text{--}1.3\ \mu\text{F}/\text{cm}^2$ and the resistance across the membrane is on the order of $10^2\text{--}10^5\ \Omega\cdot\text{cm}^2$.³⁶ The adsorption of bacteria or their metabolites to the electrode reduces its effective surface area and, hence, an increase in impedance is detected. Impedimetric measurements can also be classified as a function of the bioreceptors – antibody-based sensors, nucleic acid-based sensors, bacteriophage-based sensors and lectin-based sensors. Amongst all sensor types, those based on impedance spectroscopy have been the most popular bio-recognition elements due to their sensitivity and selectivity.^{37,38}

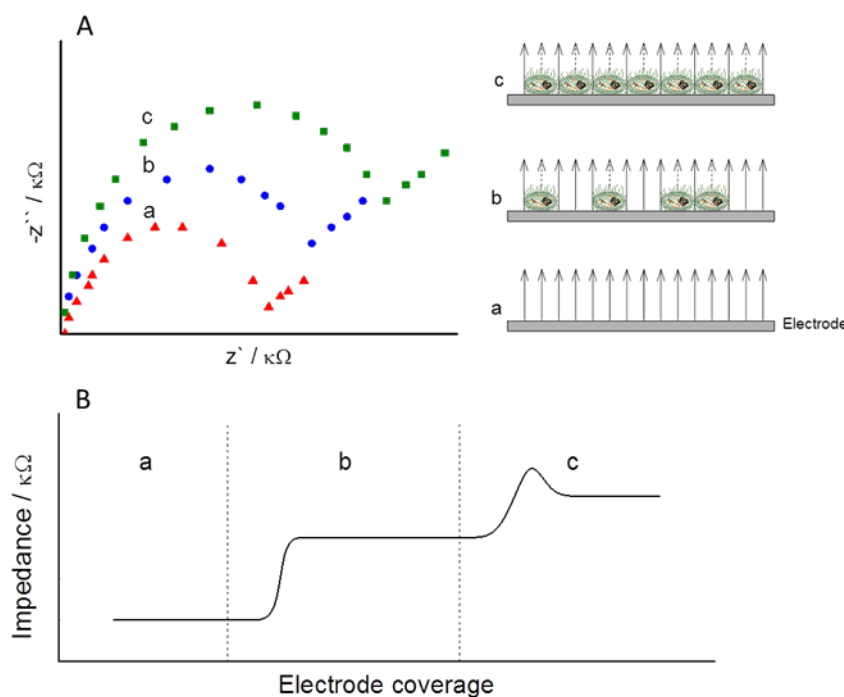


Figure 4: (A) Electrode impedance changes upon bacterial adsorption: (B) Impedance evolution versus electrode coverage. a) Plain electrode, minimum impedance; b) bacteria starting to attach to the electrode, conductivity is lowered on that surface (dashed arrows); c) maximum impedance after full coverage.

Likewise, impedimetric methods based on the detection of metabolites produced by bacterial cells as a result of growth (Figure 5) have been developed as a method that can detect bacteria within minutes.

A significant improvement in the development of high-performance impedimetric bacteria biosensors has taken place during the last few years due to the optimisation of nanoelectrodes and microfluidics.³⁷⁻⁴⁰ Micro and nanoelectrode arrays present advantages in terms of low ohmic drop (the difference in potential required to move ions throughout the solution), rapid establishment of steady-state, fast kinetics and improved signal-to-noise ratio. Other improvements include gains in the rational design and engineering of the electrolyte medium. The ideal medium should not only support the selective growth of the target bacteria, but should also provide optimal impedance signals.²⁷

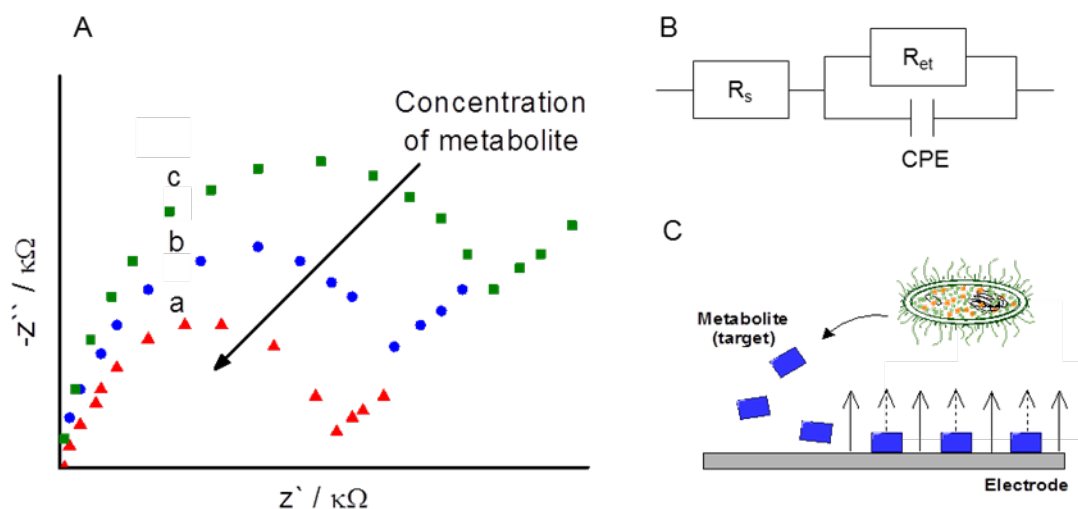


Figure 5: A) Electrode impedance changes upon secretion of metabolites by the bacteria.

B) Circuit model describing the system: solution resistance (R_s), electron transfer resistance (R_{et}) and constant phase element (CPE). C) Representation of metabolite secretion and its adsorption onto the electrode surface.

3. Case studies of electrochemical detection of pathogens

The literature on electrochemical detection of pathogens is extensive and, therefore, not feasible to summarise in a single review. In this section we present a selection of recent and relevant reports on the development of electrochemical biosensors, selecting examples that highlight the best analysis times, sensitivity and specificity achieved thus far with electrochemical biosensors. It is important that we make a clear distinction between the electrochemical technique used by a specific device and the technologies incorporated alongside (*e.g.*, magnetic NPs, captured antibodies and enzymatic amplification); while the former has its own intrinsic advantages (Section 2.1-2.3), the latter can be combined to achieve improved specificity, low detection limits and analysis of complex sample matrices. Moreover, the use of captured biomolecules is the only means to endow these sensors with pathogen as well as strain specificity. Therefore, the purpose of this section is to demonstrate how the two components of these devices (*i.e.*, the electrochemical method and the coupled technologies) can be combined to meet the requirements of its final application. Finally, we provide a table comparing the examples given herein, summarising their detection limits, time of analysis, selectivity and overall performance.

3.1. Cases studies of voltammetric detection of pathogens

Fernandes *et al.* reported a remarkably sensitive electrochemical sensor based on DNA hybridisation and voltammetric transduction, which uses working electrodes decorated with multiwalled carbon nanotubes and chitosan–bismuth complexes for signal amplification.⁴¹ This biosensor was able to detect *Aeromonas spp.* at 1.0×10^{-14} M *aer* gene concentration - that is, 10 CFU (colony-forming units)/mL - with excellent strain differentiation and

negligible response for *E. coli*, *Staphylococcus aureus* and non-amplified *Aeromonas spp.*

Unfortunately, besides the extraordinary sensitivity and rapid electrochemical response, this protocol still requires undesirable sample pre-treatment steps including a 16-hour culture, DNA extraction and polymerase chain reaction amplification.

Another example of the voltammetric detection of pathogens was described by Bellin *et al.* using a multiplexing system displaying high sensitivity, thanks to integrated amplifiers.²⁹ The authors developed a novel colony imaging chip based on the voltammetric quantification of *P. aeruginosa*'s metabolites. This system consisted of an array of 60 thin film gold working electrodes distributed in 5 channels, each containing integrated transimpedance amplifiers multiplexed into 12 working electrodes (Figure 6). The electrochemically measured concentrations of phenazines (a redox-active metabolite produced by *P. aeruginosa*) at individual electrodes in the on-chip array can serve as a spatially resolved image of the distinctive phenazine production throughout the colony. Using SWV, the authors identified and quantified (for concentrations as low as 2.6 μM) four distinct redox-active metabolites (phenazines) produced in both wild-type and mutant *P. aeruginosa* PA14 biofilms. This system exemplifies the potential applications of miniaturised and multiplexed electrochemical sensors, capable of giving specific chemical and space resolved information beyond simple pathogen detection. However, this device still requires the growth of *P. aeruginosa* on agar plates and seems more useful for research purposes in microbiology laboratories than for on-site detection of pathogens in clinical and environmental samples.

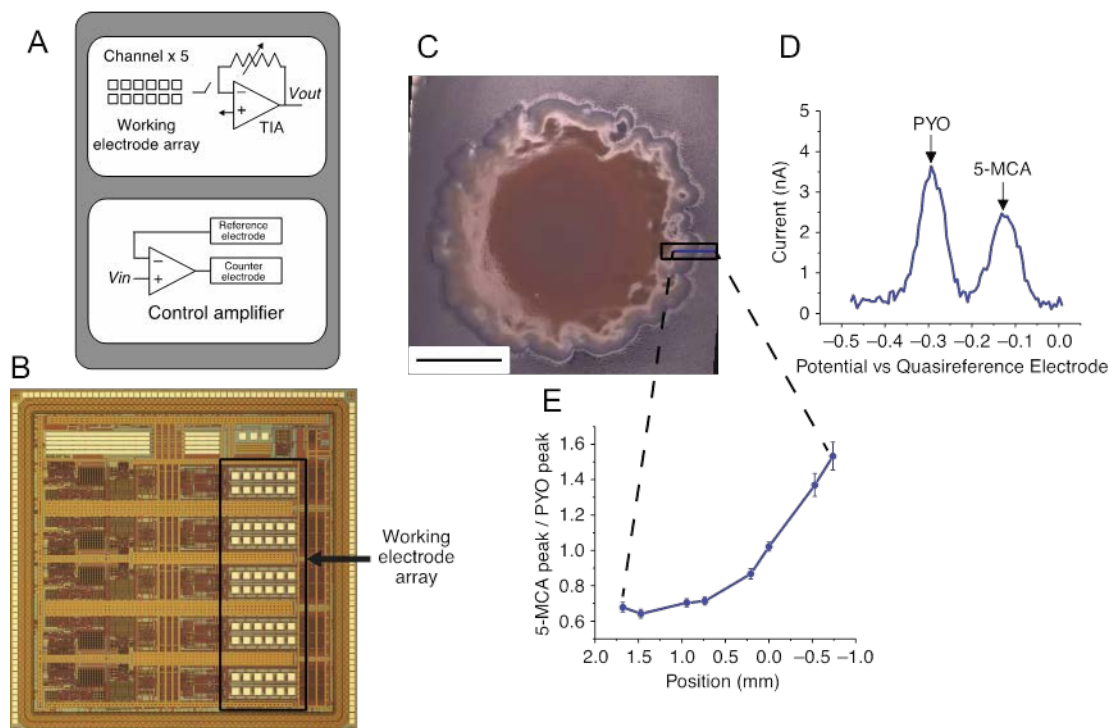


Figure 6: (A) Block diagram of the integrated circuit. (B) Optical micrograph of the integrated circuit with the working electrode array highlighted. Scale bar, 1 mm. (C) Region within a *P. aeruginosa* PA14 BigBlue9 colony biofilm that is electrochemically imaged. The black box represents the field of view of the integrated circuit, and the blue line represents the column of pixels (electrodes) probed. Scale bar, 5 mm. (D) Example of a baseline-subtracted, positive-to-negative square wave voltammogram from a single electrode, showing pyocyanin (PYO) and 5-methylphenazine-1-carboxylic acid (5-MCA) (or a derivative thereof) current peaks as a function of the applied potential versus the quasireference electrode. (E) Ratio of 5-MCA to PYO concentration along one pixel column for the imaged region shown in (C). Reprinted with permission from ref.²⁹

A similar circuit chip system of 100 working electrodes with 30 off-chip contacts and 5 separate liquid channels was implemented by Lam *et al.* in the analysis of *E. coli* and *S.*

aureus (Figure 7).⁴² The micron-sized electrodes reported by the authors exhibit an increased cross-section for the interaction with the analyte, whereas the nanostructuring enhances the sensitivity by improving the hybridisation efficiency between the tethered probe and the analyte. The patterned microsensors are functionalised with peptide nucleic acid probes specific to regions of the targeted pathogens. To detect positive target binding, the system relies on the redox behaviour of $\text{Ru}(\text{NH}_3)_6^{3+}$ and $\text{Fe}(\text{CN})_6^{3-}$. $\text{Ru}(\text{NH}_3)_6^{3+}$ is electrostatically attracted to the phosphate backbone of nucleic acids bound near the surface of the electrodes by probe molecules, and is then reduced to $\text{Ru}(\text{NH}_3)_6^{2+}$ when the electrode is biased at the reduction potential. The $\text{Fe}(\text{CN})_6^{3-}$, present in solution, oxidises $\text{Ru}(\text{NH}_3)_6^{2+}$ back to $\text{Ru}(\text{NH}_3)_6^{3+}$, which allows for multiple turnovers of $\text{Ru}(\text{NH}_3)_6^{3+}$ and generates an electrocatalytic current. The difference between the rehybridization and post-hybridization currents are used as a metric to determine positive target binding. The multiplexed system showed high selectivity in clinical samples contaminated with relevant concentrations (1 CFU/ μL) of *E. coli* and *S. aureus* and the response time was 2-5 minutes.



Figure 7 – The solution circuit chip: (A) A solution circuit chip featuring 5 liquid channels containing 20 sensors each. (B) A solution circuit chip featuring common working electrodes, and counter and reference electrode pairs. (C) An optical image of single probewell with 4 working electrodes. (D) A cross-section, looking down the liquid channel of a sensor on a solution circuit chip: glass substrate (light grey), common working electrode (yellow), SU-8 (negative photoresist) passivation/aperture layer (dark grey), counter and reference electrodes (red), SU-8 probewells (green) and SU-8 liquid channel barriers (blue). (E) Sensor-to-sensor comparison of SEM images and acid stripping scans for 20 sensors. (F) Electrochemical nucleic acid assay scheme. Peptide nucleic acids are immobilised on microsensors, and, in the presence of a complementary target, the electrostatic charge on the sensors is increased. This change in charge is read out in the presence of $\text{Ru}(\text{NH}_3)_6^{3+}$ and $\text{Fe}(\text{CN})_6^{3-}$. Reprinted with permission from ref.⁴²

Setterington *et al.* reported a fast and very sensitive electrochemical detection technique based in the combination of cyclic voltammetry with immunomagnetic separation for *Bacillus cereus* and *E. coli* O157:H7. The use of immunomagnetic beads simplifies bacteria isolation and sample pre-treatment, thus improving sensitivity. Magnetic Fe₂O₃/polyaniline core/shell NPs are employed to extract pathogens from the sample and concentrate the analyte in a single easy step. The complex pathogen-core/shell NPs is magnetically positioned on a screen-printed carbon electrode (SPCE). The current of the electrode covered with core@shell NPs decreases when the electrode is covered with the pathogen-core/shell NPs complex. After 65 min of analysis, this method showed detection limits of 40 CFU/mL and 6 CFU/mL for *B. cereus* and *E. coli* O157:H7, respectively. Despite the remarkable sensitivity of this device, no direct signal-to-concentration correlation was found and hence no quantitative data could be extracted – presumably due to the limited efficiency of the NP-electrode interface for capturing these microbes.⁴³

The virulence of some bacterial populations is regulated by the production of specific quinolones involved in cell-to-cell signalling. These metabolites offer a specific and population-dependant means to detect infection and to prevent an outbreak. Zhou *et al.* developed a sensitive and selective method to qualitatively identify *P. aeruginosa* by detecting its signal 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas Quinolone Signal, PQS). A metabolic intermediate of PQS and *Burkholderia pseudomallei*'s signal 2-heptyl-4-quinolone (HHQ) was used as specificity control, along with synthetic PQS analogs. Using cyclic voltammetry, they were able to detect PQS in the presence of HHQ on a boron-doped diamond electrode due to specific voltammetric signals associated with each metabolite.

Moreover, quantification of these metabolites was additionally performed by amperometric techniques, establishing an exceptional detection limit of 1.0 nM for PQS.⁴⁴

3.2. Cases studies of amperometric and potentiometric detection of pathogens.

J.Gau *et al.* reported an amperometric system for the detection of *E. coli* based on the integration of DNA hybridisation and enzyme amplification. Figure 8A shows how target rRNA from *E. coli* is captured by single-stranded DNA (ssDNA) attached on the electrode (via biotin-streptavidin interaction), to be then probed with detector ssDNA-fluorescein conjugate and finally coupled to anti-fluorescein-labelled peroxidase. Subsequent peroxidase-catalysed redox reactions between 3,3',5,5'-tetramethylbenzidine and H₂O₂ (Figure 8B) allowed multiple redox cycles per rRNA molecule attached and, hence, allowed for response amplification. High specificity was found towards *E. coli* stems from the distinct rRNA hybridisation. In addition, high sensitivity was accomplished by using enzymatic amplification with peroxidase (Figure 8C). Such a system has shown a response time of 40 min and a detection limit of 10³ *E. coli* cells.⁴⁵

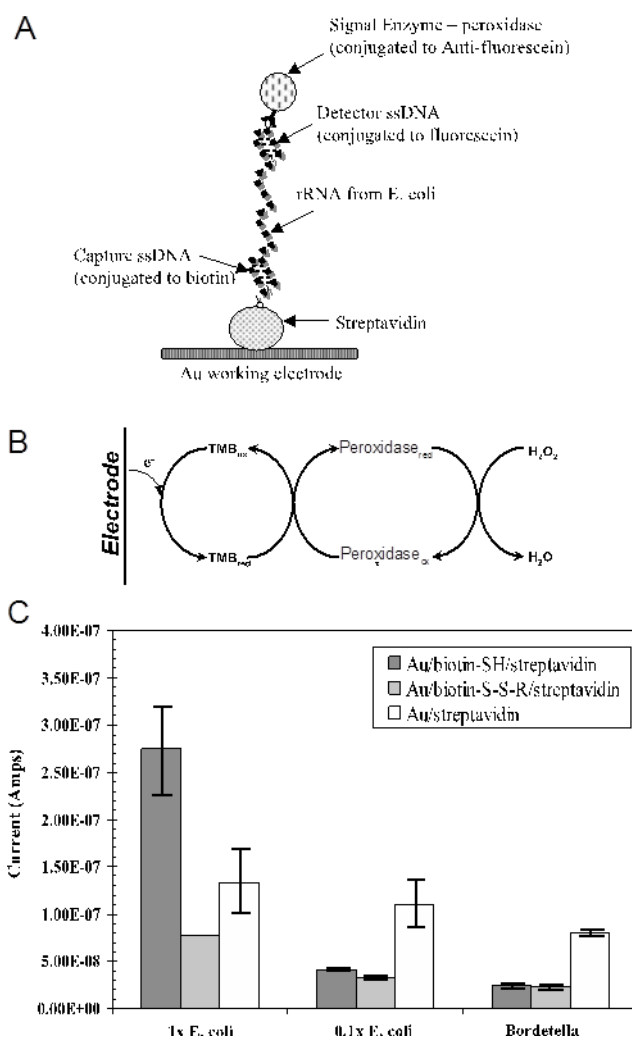


Figure 8: (A) Diagram of the modified electrode at the amperometric detection system. (B) Redox reactions occurring at the working electrode surface. The amperometric signal measured is from the reduction of the oxidised mediator (TMB). (C) Amperometric signal (reduction current at -0.1 V , $t=20\text{ s}$) as a function of a number of bacterial cells using streptavidin/biotin-SH/gold on the working electrode surface to capture the biotin-rRNA- peroxidase hybrid. The concentration of the peroxidase used was 0.15 U/mL . TMB: 3,3',5,5'-tetramethylbenzidine. Reprinted with permission from ref.⁴⁵

Lin *et al.* reported the fabrication of disposable, screen-printed carbon electrode (SPCE) strips for the rapid immunoamperometric detection of *E. coli* O157:H7.⁴⁶ The method uses

an indirect sandwich enzyme-linked immunoassay with double antibodies immobilised at the working electrode (Figure 9A). The SPCEs consist of a 9.8 mm² carbon surface working electrode and a 9.8 mm² carbon surface counter/reference electrode. The results show that the combined use of carbon electrodes modified with gold NPs and of ferrocenedicarboxylic acid as the substrate for hydrogen peroxidase mediator enhanced the response current 13-fold. Concentrations of *E. coli* O157:H7 from 10² to 10⁷ CFU/mL were detected, and the limit of detection after 60 min was approximately 6 CFU/strip in phosphate buffered saline and 50 CFU/strip in milk. Additionally, the current response for other species was the same as that of a blank with no bacteria (Figure 9B). Key features of this sensor are the ability to analyse real food samples, portability, specificity and a wide linear response window (5.75 × 10² to 5.75 × 10⁷ CFU/mL) for detecting *E. coli* O157:H7.

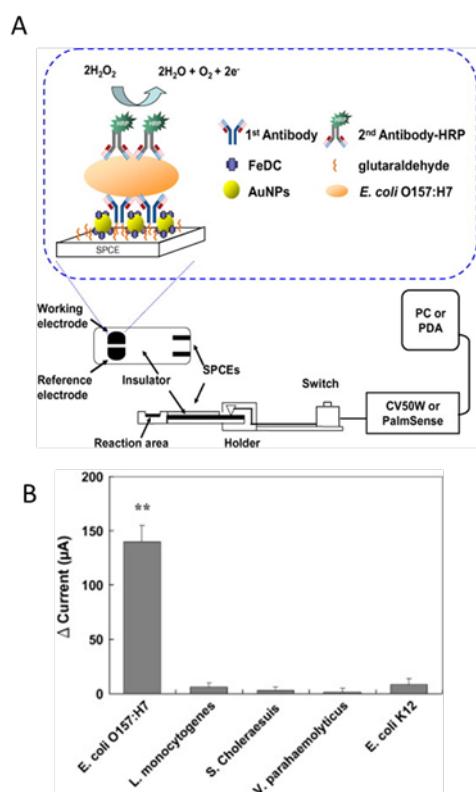


Figure 9: (A) Schematic illustration of the device and immunosensing processes of the AuNPs/FeDC–SPCE immunosensor system for *E. coli* O157:H7 detection. (B) Species and strain specificity of the amperometric detection using the AuNPs/FeDC–SPCE immunosensing strip. The various bacteria cultures – *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella choleraesuis*, *Vibrio parahaemolyticus* and *E. coli* K12 – each at a concentration of around 5×10^7 CFU/mL. HRP: horseradish peroxidase. FeDC: ferrocenedicarboxylic acid. Reprinted with permission from ref.⁴⁶

An electrochemical sensor coupled with filtration capture for the rapid detection of *E. coli* O157:H7 has been developed by Brewster and Mazenko.⁴⁷ This system uses a cellulose acetate filter to capture the enzyme-labelled antibody complex with bacteria. The filter is then put into contact with the electrode surface and the cells are detected by the

conversion of a substrate (*p*-aminophenyl phosphate) to an electroactive product (*p*-aminophenol) whose subsequent oxidation on the working electrode is quantified amperometrically. Thus, the readout signal is amplified both enzymatically and by the close proximity of the deposited sample onto the electrode. The limit of detection of the method is 5×10^3 cells/mL in an assay time of 25 min.

Gehring *et al.* have used antibody-coated magnetic beads, sandwiched with an alkaline phosphatase-labelled antibody, to capture and detect *Salmonella typhimurium*.⁴⁸ A magnetic field is used to curb the beads onto the surface of a disposable graphite ink electrode. Cells are detected by the oxidation of the electroactive enzyme product. Even though this approach offers a limit of detection of 8×10^3 cells/mL in buffer, the analysis time goes up to 80 min. Liébana *et al.* implemented a similar methodology to detect *Salmonella* in milk.²³ The authors preconcentrated the bacteria from milk samples by using magnetic beads through an immunological reaction. In a second step, a polyclonal antibody, labelled with peroxidase, is used as serological confirmation with electrochemical detection based on a magneto-electrode (Figure 10). This method, with an overall analysis time of 50 min, displayed excellent microbe specificity in the analysis of *Salmonella* samples contaminated with *E. coli*, yet the limit of detection (LOD) was rather high from direct analysis of milk samples (7.5×10^3 CFU/mL).

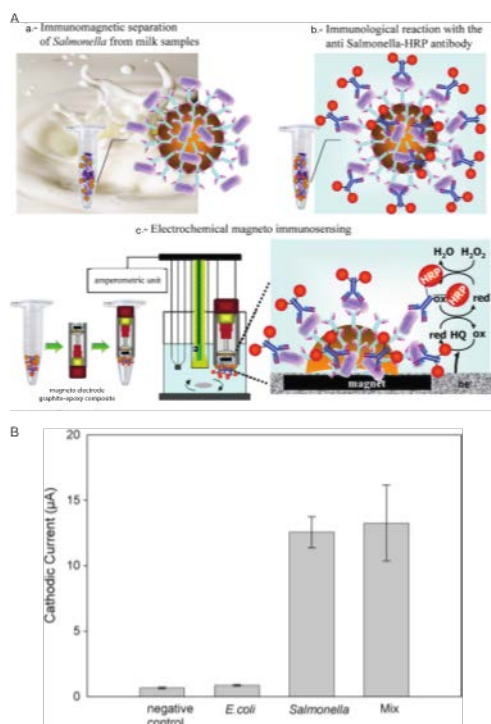


Figure 10: (A) Diagram of the 'immunomagnetic / graphite-epoxy composite electrochemical immunosensing' approach. (B) Electrochemical signal for milk diluted 1/10 in Lysogeny broth and then artificially inoculated, respectively, with: 0 CFU/mL (negative control); 2.8×10^6 CFU/mL of *E. coli*; 5.2×10^6 CFU/mL of *Salmonella*; and a mix solution containing 1.4×10^6 CFU/mL of *E. coli* and 4.65×10^6 CFU/mL of *Salmonella* spp. In all cases, 10 µL of commercial anti-*Salmonella* magnetic beads were used, as well as anti-*Salmonella*-horseradish peroxidase antibody, diluted 1/1000 in Lysogeny broth. The electrochemical detection was performed in phosphate buffered saline containing 1 mM ethylenediaminetetraacetic acid. Mediator: hydroquinone (HQ) 1.81 mM. Substrate: H_2O_2 4.90 mM. Applied potential = -0.100 V (vs. Ag/AgCl). HRP: horseradish peroxidase.

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Another selective detection method was described by Rishpon and Ivnitski using a separation-free amperometric enzyme-channelling immunosensor for the detection of *S. aureus*.⁴⁹ A carbon electrode was modified both with glucose oxidase and an antibody capable of binding the antigen of interest (protein A from *S. aureus*). Experiments were done in the presence of an antigen-horseradish peroxide conjugate, so that upon antibody-antigen binding, glucose oxidase and horseradish peroxidase were brought into contact. This contact triggered the oxidation of glucose to yield H_2O_2 , which was used by horseradish peroxidase to oxidise iodide. This catalytic cycle was perturbed in the presence of exogenous antigen, allowing the detection of *S. aureus* to a limit of 10^3 cells/mL of pure culture in 30 min. A key feature of this system was the use of electrodes coated with poly(ethylene imine), which minimised unspecific binding of sample components to the electrode surface, and hence, reduced the background signal.

Electrochemical immunosensors can also be improved by the use of flow injection systems, as was demonstrated by Pérez *et al.*⁵⁰ Flow-injection immunofiltration increases the ratio of surface area of immunosorbent to sample volume, offering increased antibody–antigen interaction. The authors implemented an amperometric flow-injection sensor with immunomagnetic separation for the detection of *E. coli* O157:H7 cells. Such detection was performed by measuring the oxidation of two mediators ($Fe(CN)_6^{3-}$ and 2,6-dichlorophenolindophenol) in phosphate-buffered saline solutions (with KCl). The lower detection limit was 10^5 CFU/mL in a time of 2 h, with pre-enrichment necessary for low numbers of bacteria. This method has the advantages of only detecting viable cells – as it monitors cell metabolism – and of only requiring a single and non-labelled capture antibody, making the

assay faster and less expensive compared to enzyme-linked immunosorbent assay-based technologies.

Sensitivity of electrochemical immunosensing techniques can be improved at all stages of signal transduction (*e.g.*, enzymatic reactions and electrochemical amplification). Chemburu *et al.* reported a remarkably sensitive amperometric system for microbe detection based on dual-action carbon NPs coupled in a sandwich immunoassay. Antibody-functionalised carbon NPs were used to selectively adsorb bacteria from complex samples by filtration. Bacteria-coupled NPs were then connected to the electrochemical circuit and used as working electrode. The use of porous carbon NPs as working electrodes offered higher surface area and therefore sensitivity compared to solid electrodes. Subsequent incubation with horseradish peroxidase-labelled antibodies allowed measurement of enzymatic conversion of 3,3',5,5'-tetramethylbenzidine, a substrate 100 times more sensitive than those previously described. The improvements in both working electrode surfaces and enzymatic reaction efficiency allowed sensing of *E. coli*, *Listeria* and *Campylobacter* from milk and chicken samples, with detection limits of 50, 10 and 50 cells/mL in 40 min, respectively.⁵¹

Highly-sensitive potentiometric detection of pathogens was reported by Zelada-Guillén *et al.*, where single-walled carbon nanotubes were decorated with specific aptamers against *E. coli* CECT 675. Almost real-time sensing was achieved from complex samples with detection limits of 6 CFU/mL in milk and 26 CFU/mL in apple juice. Binding of *E. coli* to capture aptamers was recorded as variations in the potential of the electrochemical circuit. Both interstrain and interspecies selectivity was observed versus *E. coli* CECT 4558, *Salmonella* and *Lactobacillus*. Moreover, this sensor was able to be regenerated by a simple wash with

2M NaCl and it tolerated up to five regeneration cycles without any effects on sensitivity or instrumentation noise. Linear response in detection was found in concentrations of up to 10^4 CFU/mL.⁵²

3.3. Cases studies of impedimetric detection of pathogens.

Huang *et al.* reported a novel immunosensor based on antibody-functionalised Fe₃O₄ NPS modified with O-carboxymethylchitosan for the rapid detection of *Campylobacter jejuni*.⁵³ This biosensor showed a wide linear detection range of 10^3 to 10^7 CFU/mL in which the measured changes of impedance were proportional to the logarithmic value of *C. jejuni* concentration, yielding a limit of detection of 10^3 CFU/mL. This detection tool presented some advantages such as easy regeneration of the immunosensor, high selectivity and negligible interference from *E. coli* and *Salmonella sp.* However, even though the electrochemical detection was considerably fast, the sample preparation was time-consuming because bacteria had to be extracted and incubated for 36 hours.

Barreiros dos Santos *et al.* reported a highly-sensitive, label-free immunosensor for the detection of pathogenic *E. coli* O157:H7.⁵⁴ Anti-*E. coli* antibodies were covalently immobilised onto gold electrodes via a self-assembled monolayer (SAM) of mercaptohexadecanoic acid, and the pathogenic bacteria were detected by electrochemical impedance spectroscopy (Figure 11A-B). Significant differences in the impedance measurement were observed upon an increase of bacteria concentrations. The immunosensor showed a very low limit of detection (2 CFU/mL) and a long linear range (from 3×10^{-3} to 10^4 CFU/mL). In the presence of *S. typhimurium* and *E. coli* O157:H7, this

biosensor showed high selectivity to *E. coli* O157:H7 with negligible response to *S. typhimurium* adsorption (Figure 11C).

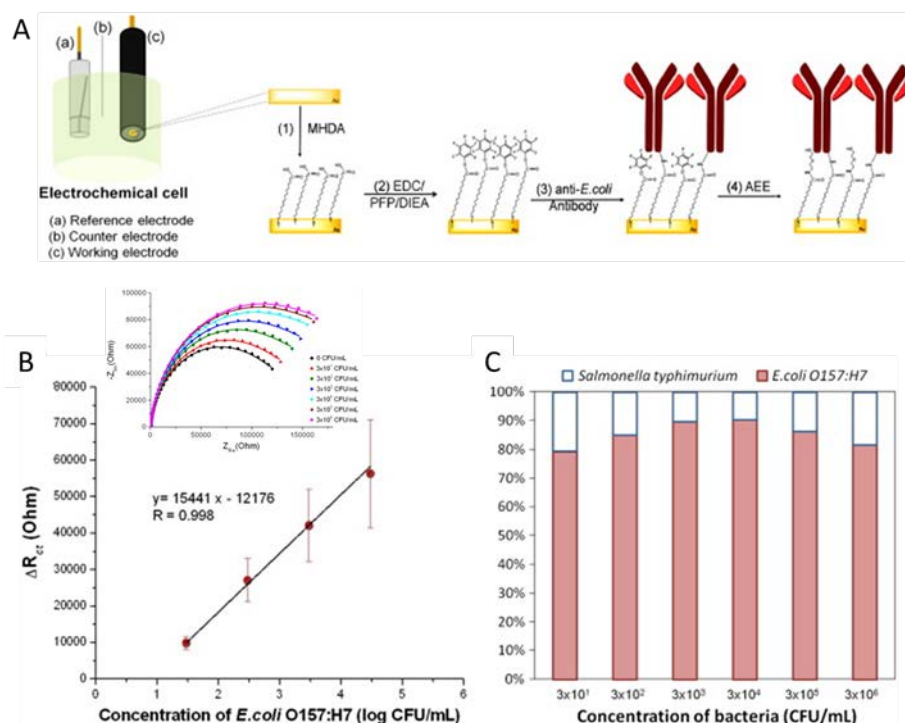


Figure 11: (A) Diagram for the immunosensor fabrication: (1) acid-terminated self assembled monolayer, (2) surface activation, (3) antibody binding and (4) chemical blocking. (B) Relationship between changes of the impedance as a function of the concentration of *E. coli* O157:H7 with its corresponding fitting. (C) Normalised change of the impedance between *E. coli* O157:H7 and *S. typhimurium* at different concentrations. EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, PFP: pentafluorephenol, DIEA: diisopropylethylamine, AEE: 2-(2-aminoethoxy)ethanol. Reprinted with permission from ref.⁵⁴

An impedimetric biosensor, based on an interdigitated array microelectrode coupled with magnetic NP–antibody conjugates, was reported by Varshney *et al.*⁵⁵ The authors

implemented this system for the detection of *E. coli* O157:H7 in ground beef samples.

Biotin-labelled anti-*E. coli* antibodies, immobilised onto streptavidin-coated magnetic NPs, were used to separate and concentrate *E. coli* O157:H7 from ground beef samples. The limit of detection of the biosensor for *E. coli* O157:H7 in ground beef samples was 8.0×10^5 CFU/mL and the detection time of the measurement was 35 min.

Shabani *et al.* reported a novel specific detection method of *E. coli* K12 using modified SPCEs with bacteriophages. Carbon electrodes were functionalised with N-ethyl-N'-(3-dimethylaminopropyl) ethylcarbodiimide and then coupled with a T4 phage, forming an amide bond. In the presence of the bacteria, the impedance measurements presented a shift of $10^4 \Omega$ due to bonding between the bacteria and the T4 phage. The limit of detection was established in 10^4 CFU/mL for 50 μ L samples, yielding a potential approach for specific bacteria detection.⁵⁶ In addition, in order to demonstrate the specificity of the system for the detection of *E.coli*, control impedimetric measurements were performed in presence of *S. typhimurium*. As a result, non-significant changes associated with the phage capture of *S. typhimurium* were observed in the impedimetric signal.

4. Conclusions and perspectives

Table 1. Summary of cases studies: methodologies, performance and sensitivities.

Transduction	Coupled to	Comments	Analyte	LOD	Analysis time
Voltammetry	DNA hybridisation MWCNT	<ul style="list-style-type: none"> Specificity PCR amplification Sensitivity 	<i>Aeromonas spp.</i> (DNA)	10^{-14} M	>16 h ⁴¹

		<ul style="list-style-type: none"> CV/DPV/IS 			
	60-electrode array	<ul style="list-style-type: none"> Colony imaging SWV 	<i>P. aeruginosa</i> (metabolites)	2.6 μ M	4 min ²⁹
	100-electrode array DNA hybridisation	<ul style="list-style-type: none"> High-Throughput Specificity CV/DPV 	<i>E. coli</i> <i>S. aureus</i>	1 CFU/mL	2-5 min ⁴²
	IMNP	<ul style="list-style-type: none"> Specificity Analyte isolation Sensitivity CV 	<i>E. coli</i> <i>B. cereus</i>	6 CFU/mL 40 CFU/mL	65 min ⁴³
	N/A	<ul style="list-style-type: none"> Simplicity Sensitivity CV 	<i>B. pseudomallei</i> (metabolites)	1 nM	30 min ⁴⁴
Amperometry/ Potentiometry	Enz. Amplification DNA hybridisation	<ul style="list-style-type: none"> Sensitivity Gene specificity 	<i>E. coli</i>	10 ³ cells	40 min ⁴⁵
	SPCE Antibody Enz. amplification	<ul style="list-style-type: none"> Portability Sensitivity 	<i>E. coli</i>	6* and 50** CFU/ strip	60 min ⁴⁶
	Cellulose filters Antibody Enz. amplification	<ul style="list-style-type: none"> Analyte isolation Sensitivity 	<i>E. coli</i>	5 \times 10 ³ cells/mL	25 min ⁴⁷
	IMNP	<ul style="list-style-type: none"> Analyte isolation 	<i>Salmonella sp.</i>	8 \times 10 ³	80 min ⁴⁸

	Enz. amplification	<ul style="list-style-type: none"> Sensitivity 		cells/mL	
	IMNP Enz. amplification	<ul style="list-style-type: none"> Specificity Analyte isolation Sensitivity 	<i>Salmonella sp.</i>	7.5×10^3 cells/mL	50 min ²³
	Antibody	<ul style="list-style-type: none"> Separation-free Disposable cells 	<i>S. aureus</i>	10 ³ cells/mL	30 min ⁴⁹
	IMNP Metabolic sensing	<ul style="list-style-type: none"> Analyte isolation Viable cells only 	<i>E. coli</i>	10 ⁵ CFU/mL	2 h ⁵⁰
	Carbon NPs Antibody Enz. amplification	<ul style="list-style-type: none"> Sensitivity Specificity 	<i>E. coli</i> <i>Listeria sp.</i> <i>Campylobacter</i> sp.	50 cell/mL 10 cell/mL 50 cell/mL	40 min ⁵¹
	Aptamers SWCNT	<ul style="list-style-type: none"> Electrode reuse Specificity Sensitivity 	<i>E. coli</i>	6 CFU/mL ^{**} 26 CFU/mL ^{***}	Real time ⁵²
Impedance	Antibody	<ul style="list-style-type: none"> High selectivity Immunosensor regeneration No PCR 	<i>C. jejuni</i>	1×10^3 CFU/mL	40 min ⁵³
	GE	<ul style="list-style-type: none"> Increased 	<i>E. coli</i>	2 CFU/mL	45 min ⁵⁴

		sensitivity <ul style="list-style-type: none"> Improved selectivity Non sample pre-treatment 			
	IDAM-MNAC	<ul style="list-style-type: none"> Fast bacteria separation Fast response time Non sample incubation, redox probes and immobilization techniques 	<i>E. coli</i>	8×10^5 CFU/mL	35 min ⁵⁵
	Bacteriophages SPCE	<ul style="list-style-type: none"> High selectivity Specificity Electrode incubation 	<i>E. coli</i>	10^4 CFU/mL	25 min ⁵⁶

MWCNT: Multiwalled carbon nanotube; MNP: Magnetic nanoparticle; PCR: Polymerase chain reaction; IMNP: Immunomagnetic nanoparticles; CV: Cyclic voltammogram; DPV: Double pulse voltammetry; SWV: Square wave voltammetry; EIS: Electrochemical impedance spectroscopy; LOD: Limit of detection; SPCE: Screen-printed carbon electrode; IDAM-MNAC: Interdigitated array microelectrode-magnetic nanoparticle–antibody conjugates. *In buffer, **In milk, ***In juice.

The continuous and significant activity in the development of electrochemical sensors is a clear indication of the importance of the field and of the potential of electrochemical methods to provide rapid and straightforward solutions to the field of biosensing.

Electrochemical biosensors offer a very sensitive (both qualitatively and quantitatively), inexpensive and time saving alternative to traditional culturing methods. In most cases, these sensing platforms provide “real-time” and highly-selective multiple analyses without pre-enrichment or pre-concentration steps that are essential for the *in situ* detection of bacteria in food and water.

Electrochemical biosensors have been investigated for over 10 years, but despite enormous advances that have been already achieved, the penetration of biosensors into the market is slow. This is a consequence of the different challenges that this technology is still facing – optimisation in sample preparation, in analysis time and in device sensitivity. Therefore the progress of electrochemical sensors for the detection of pathogens requires a significant effort in fundamental, technical and mechanistic studies.

In particular, the development of novel nanomaterials with high surface area, conductivity and stability would bring about the essential performance upgrades necessary for the ultimate commercialisation of these sensors. Moreover, the progress in the design of innovative and low cost ligands (*e.g.*, synthetic (glycol)polymers), with higher selectivity and stability, will help to define new biorecognition elements for improved biosensors.

New advances in the development of sensors with microfluidics, transducer sensitivity, signal amplification, magnetic filtration, microelectrode arrays and antibody design and production, have provided significant improvements to the sensitivity and the selectivity targets in the detection of pathogens. In addition, these advances represent a step forward

towards the design of fully-automated, portable and inexpensive biosensors for the rapid detection of microorganisms both on-field and in the laboratory.

Beside all of these technological advances, electrochemical sensors with even higher sensitivity are limited by the residence time of the analyte with the electrochemical interface. Intensive efforts should be made to increase the mass transport of the analyte towards the electrochemical interface (via microfluidic systems or localised increases of temperature) and/or to form a homogenous distribution of the sample at the electrochemical interface (via conductive high-surface-area nanoporous sol-gels and aerogels).

In terms of the engineering, electronic and computational aspects, one of the great challenges of electrochemical nanosensing is the development of fully-automated microarray platforms with rapid electronic response for data analysis and display. This, however, requires the development of improved mathematical models that enable multianalyte detection.

Finally, intensive research should focus on validating these biosensors on large populations of real samples. These studies should include complex clinical and environmental samples with assorted matrices, instead of using laboratory standards, as to do so will ensure response robustness and reproducibility.

In summary, ideal electrochemical biosensors should be sensitive, portable, fast and easy to use by non-specialists without compromising the accuracy of the analysis. Further work is necessary for the wide commercialisation of cheap and long storage devices with minimum sample pre-treatment. Overcoming all these challenges will bring electrochemical

biosensors to the general user, providing a powerful tool for early diagnosis, outbreak prevention and tackling the spread of infection.

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